

Antifungal potential of entomopathogenic bacteria, *Photorhabdus*, and *Xenorhabdus* (Morganeliaceae) against pathogenic fungi

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ABSTRACT

Entomopathogenic bacteria (EPB) are natural pathogens of insects being utilized as biological control agents for insect pests worldwide. In addition to their pathogenicity against insects, they are known to inhibit the growth of several microbes. In this study, EPB of the genus, *Photorhabdus* and *Xenorhabdus*, were investigated for their pathogenicity against *Fusarium solani* (CCK3A1), *Fusarium keratoplasticum* (ATCC 36031), *Candida albicans* (ATCC 2091), and *Aspergillus fumigatus* (ATCC 204305). The antagonistic effect of cell suspension was analyzed by calculating percent inhibition from the co-cultured plate of bacteria and fungus within 192 hours of incubation at 25°C where the highest percent inhibition was observed with *X. vietnamensis* (RF) against *A. fumigatus* (ATCC 204305). Moreover, the activity of ethyl acetate extract of bacterial metabolites against pathogenic fungi was analyzed using the disk diffusion method where *Photorhabdus hindustanensis* (TS) isolates exhibited the highest inhibition against *A. fumigatus* (ATCC 204305). The components of ethyl acetate extract were analyzed using gas chromatography–mass spectrometry in which Pyrrolo [1,2-a] pyrazine-1,4-dione hexahydro-3-(phenylmethyl), benzenecetic acid, and n-Hexadecanoic acid were found to be the most abundant compounds. This study provides information regarding the potential of *Photorhabdus* and *Xenorhabdus*, including their secondary metabolites against several pathogenic fungi. It further provides insights to overcome the current global drug resistance crisis among several pathogenic fungi, as well as new reference data for the future development and application of antifungal agents.

1. INTRODUCTION

Fungal infections are one of the most common and devastating problems worldwide. Over 150 million cases of serious fungal infections occur worldwide each year, and approximately 1.7 million people die as a result [1,2]. These estimates have continuously risen due to societal and medical improvements that have aided in spreading fungal illnesses over the years. As a result, fungal infections are becoming a global problem for human health due to increased overuse and prophylactic application of antifungals to immunocompromised individuals promoting the emergence of multi-drug-resistant fungi [1,3]. Apart from human infection, over 19,000 fungi are known to infect economically important crops worldwide accounting for over 30% of all crop diseases [4].

The genus *Fusarium*, belonging to the Nectriaceae family, is characterized as an imperfect fungus with a global distribution capable of infecting animals, plants, and humans. Keratitis, onychomycosis,

eumycetoma, skin lesions, and disseminated infections are all connected with *Fusarium* infection in humans [5]. Apart from human infection, it is a broad-spectrum fungus species that has been shown to infect peas, beans, potatoes, and different cucurbits [6]. Because of the significant death rate caused by *Fusarium* and the growing resistance to the currently used antifungal medicines (azoles), a novel therapy is required [7].

The genus *Candida* is a member of the Saccharomycetaceae family. There are currently around 150 *Candida* species, with approximately 20 species known to cause infections in humans [8]. In a healthy person, *Candida albicans* colonizes the oral mucosa, vagina, skin, as well as gastrointestinal tract asymptotically as a commensal fungus, accounting for more than 80% of asymptomatic human vaginal and oral yeast strains [9,10]. Eventually, it possesses different properties, both commensal and pathogenic, that become part of the human natural microbiome and invade tissues and organs when the immune system is impaired [11,12]. The rapid rise of various antifungal resistance among *Candida* spp. is a serious health concern worldwide and a better understanding of resistant mechanisms with the identification of a new drug target could reduce the current situation of developing resistance upon prolonged exposure to antifungal agents [13,14].

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The genus *Aspergillus*, a fungus belonging to the family Trichocomaceae, is a common mold in the environment and the leading cause of aspergillosis in people. It is a deadly pathogen that can kill people with weakened immune systems, underlying diseases, or transplants [15–17]. Also, *Aspergillus* hampers plant photosynthesis by impeding the production of carotenoid and chlorophyll, resulting in albinism or virescence in the infected plants [18]. Resistance to existing antifungal medications, azoles, emerged as a global concern in *Aspergillus fumigatus* infection with a prevalence of 6.6%–28% [19]. The prevalence of fungal disease has led to the rapid rise of resistance in economically important fungi resulting in the difficulty of effective treatment. Apart from human concerns, chemical fungicides are still the most effective and widely used for controlling fungal infections. However, because of their harmful effects on people and other nontarget creatures, they are also harmful to the environment and even pose risks to the health condition of humans [20]. Hence, biological control, which includes the employment of antagonistic organisms, including their secondary metabolites and volatile organic compounds (VOCs), is a crucial candidate and alternative agent to chemical fungicides [21].

Since the penicillin era, microbial metabolites have still been a crucial source in the development of novel drugs for animals and humans. *Photorhabdus* and *Xenorhabdus*, belonging to the family Morganellaceae, are an obligate symbiosis with entomopathogenic nematodes (EPNs) of the genus *Heterorhabditis* and *Steinernema*, respectively [22–24]. When the nematode infects the insect larvae, it releases the bacterial symbionts inside the hemocoel of the insect resulting in the production of broad-spectrum compounds; the compounds are lethal to insect larvae as well as antagonists to bacteria, fungi, nematodes, protozoa, and cancer cells [25]. The EPNs are distributed worldwide and are being utilized for the biological control of insect pests with a high success rate [26]. The secondary metabolites isolated from fermented culture media of *Photorhabdus* spp. and *Xenorhabdus* spp., including methanol and ethyl acetate extracted compounds, were found to be effective in suppressing several pathogenic bacteria [27–29] and fungi [30–32] to a great extent.

Despite technological breakthroughs in pharmaceutical manufacturing, there is still a need to develop new prospective antifungals due to the rapid development in resistance to current antifungal drugs. The antifungal activity of *Photorhabdus* and *Xenorhabdus* bacteria has garnered attention due to their potential as biocontrol agents against various phytopathogenic fungi. Both genera produce secondary metabolites that exhibit significant antifungal properties, making them valuable in agricultural practices. Therefore, this study was carried out to determine the potential of *Photorhabdus* and *Xenorhabdus* against pathogenic fungi against *F. solani* (CCK3A1), *Fusarium keratoplasticum* (ATCC36031), *C. albicans* (ATCC 2091), and *A. fumigatus* (ATCC204305). The VOCs present in the ethyl acetate extract of the bacterial isolates were also characterized using gas chromatography–mass spectrometry (GC-MS) analysis.

2. MATERIALS AND METHODS

2.1. Source of Symbiotic Bacteria

Four species of symbiotic bacteria, i.e., *Photorhabdus hindustanensis* (TS), *P. namnaonensis* (TD), *Xenorhabdus vietnamensis* (RF), and *X. stockiae* (PTS), previously isolated from their respective symbionts, *Heterorhabditis indica*, *H. baujardi*, *Steinernema sangi*, and *S. surkhetense* were used for the experiment [33]. The nematodes were locally isolated from Mizoram, India, with geographical coordinates of 22.350 N 93.060 E (*H. indica*), 23.740 N 92.952 E (*H. baujardi*), 23.370 N 93.161 E (*S. sangi*), and 22.960 N 92.612 E (*S. surkhetense*).

Fresh cultures of the bacteria were obtained by spreading a volume of 100 µl on NBTA medium (nutrient agar supplemented with 0.0025% bromothymol blue and 0.004% triphenyl tetrazolium chloride) followed by incubation at 28°C for 48 hours [34,35]. A single colony of bacteria that absorbs bromothymol blue dye was selected and streaked on nutrient agar for further characterization.

2.2. Preparation of Pathogenic Fungi

Standard-type cultured strains, i.e., *F. keratoplasticum* (ATCC 36031), *Candida albicans* (ATCC 2091), *A. fumigatus* (ATCC 204305), and *Fusarium solani* (CCK3A1), were used as pathogens for the experiment. *Fusarium solani* (CCK3A1) was locally isolated from ginger soft rot tissue and the ITS1 gene sequence was submitted to NCBI GenBank with accession number, OR793128. Before the experiment, the fungi were grown on a potato dextrose agar (PDA) (HiMedia®) and incubated at 25°C to check their purity and viability. The fresh cultures were further used for an antagonistic test. Determining the activity of ethyl acetate extract, the fungi were diluted to 0.05% Tween® 80 solution to obtain a homogenized spore suspension [36].

2.3. Antagonistic Effect of Bacterial Suspension

The antagonistic effects of *Photorhabdus* (TS and TD) and *Xenorhabdus* (RF and PTS) were performed as per Chen *et al.* [30] with a slight modification. A total of 10 ml of the 48-hour bacterial culture was spread on a Petri plate consisting of PDA. A sterile blade was used to cut out approximately 4 mm of fungal mycelia, which was subsequently collected using fine sterile forceps and inoculated on a PDA plate consisting of the bacterial symbiont. The control plate contains PDA that has no bacterial symbiont. The experiment was carried out in triplicate, and the diameter of fungal growth was measured and compared with the control plate after 48, 96, and 192 hours. The percent inhibition of the fungus was calculated by using the following formula [37]:

$$\text{Antifungal activity (\%)} = \frac{D_c - D_s}{D_c} \times 10$$

where D_c is the diameter of fungal growth in the control plate and D_s is the diameter of fungal growth in the plate containing bacterial isolates.

2.4. Preparation of Bacterial Extract

The solvent system extraction of bacterial metabolites was conducted according to Muangpat *et al.* [29] with a slight modification. A single colony was transferred to a 1,000-ml sterile nutrient broth and stored at 28°C in a shaker incubator for 48 hours. To extract the crude compound, the same volume of ethyl acetate was added and mixed well in a 2,000-ml separating funnel. The mixture was left at room temperature for 24 hours. The ethyl acetate layer was then collected followed by evaporation using a rotary vacuum evaporator (Rotavapor® R-100 System-Buchi, Switzerland). The extraction procedure was performed thrice to maximize the yield of crude extract.

2.5. Activity of Ethyl Acetate Extract

The condensed bacterial extract was adjusted to 500 mg/ml in dimethyl sulfoxide (DMSO) and kept as a stock solution. A 10-ml bacterial extract was impregnated onto 6-mm sterile disks and then placed in the center of the PDA plates comprising the homogenized fungal spores. The plates were incubated at 25°C for 48 hours depending on the growth of fungal mycelia. The diameter of the inhibition zone was

measured and expressed in millimeters. DMSO was used as a negative control and fluconazole disk was used as a positive control.

2.6. GCMS Analysis of Ethyl Acetate Extract of Bacterial Isolates

GC-MS analysis of bacterial ethyl acetate extract was carried out using GC-MS QP2010 model (Shimadzu®), Column, GC, SH-I-5Sil MS Capillary, 30 m × 0.25 mm × 0.25 µm, injection mode: splitless. The oven temperature was programmed as follows: 45°C for 2 minutes and then 140°C at 5°C/ minute and finally increased to 280°C and held isothermally for 10 minutes; 2 µl of each sample was injected and helium gas was used as a carrier gas with a flow rate of 1 ml/minute. The ionization of the sample components was carried out at 70 eV. The National Institute of Standards and Technology database, which has over 62,000 patterns, was used to describe the components of the GC-MS mass spectrum.

2.7. Statistical Analysis

Statistical analysis was performed using SPSS software (Version 20.0). The percent inhibition from the antagonistic activity and diameter of

the inhibition zone from the disk diffusion test of ethyl acetate extract was calculated and expressed as mean ± standard error (SEM). One-way analysis of variance was used to assess the variation of inhibition. The confidence level was set at 5%, indicating that the treatment of bacteria against the fungi will be significant if $p < 0.05$ with a 95% confidence interval.

3. RESULTS

3.1. Antagonistic Effect of Bacterial Isolates Against Pathogenic Fungi

The cell suspension of bacterial isolates showed inhibition of growth against pathogenic fungi within 196 hours post-inoculation. The growth inhibition of bacterial isolates against the pathogenic fungal strains and the graphical representation of percent inhibition along with a mean diameter of fungal growth were shown in Figures 1 and 2.

The bacterial isolates exhibited significant variations of activity against *F. solani* (df = 3 and 68; F = 54.64; $p < 0.05$). *Photorhabdus hindustanensis* (TS) and *P. namnaonensis* (TD) isolates exhibited growth inhibition of 48.86% and 52.17%, respectively, at 192-hour

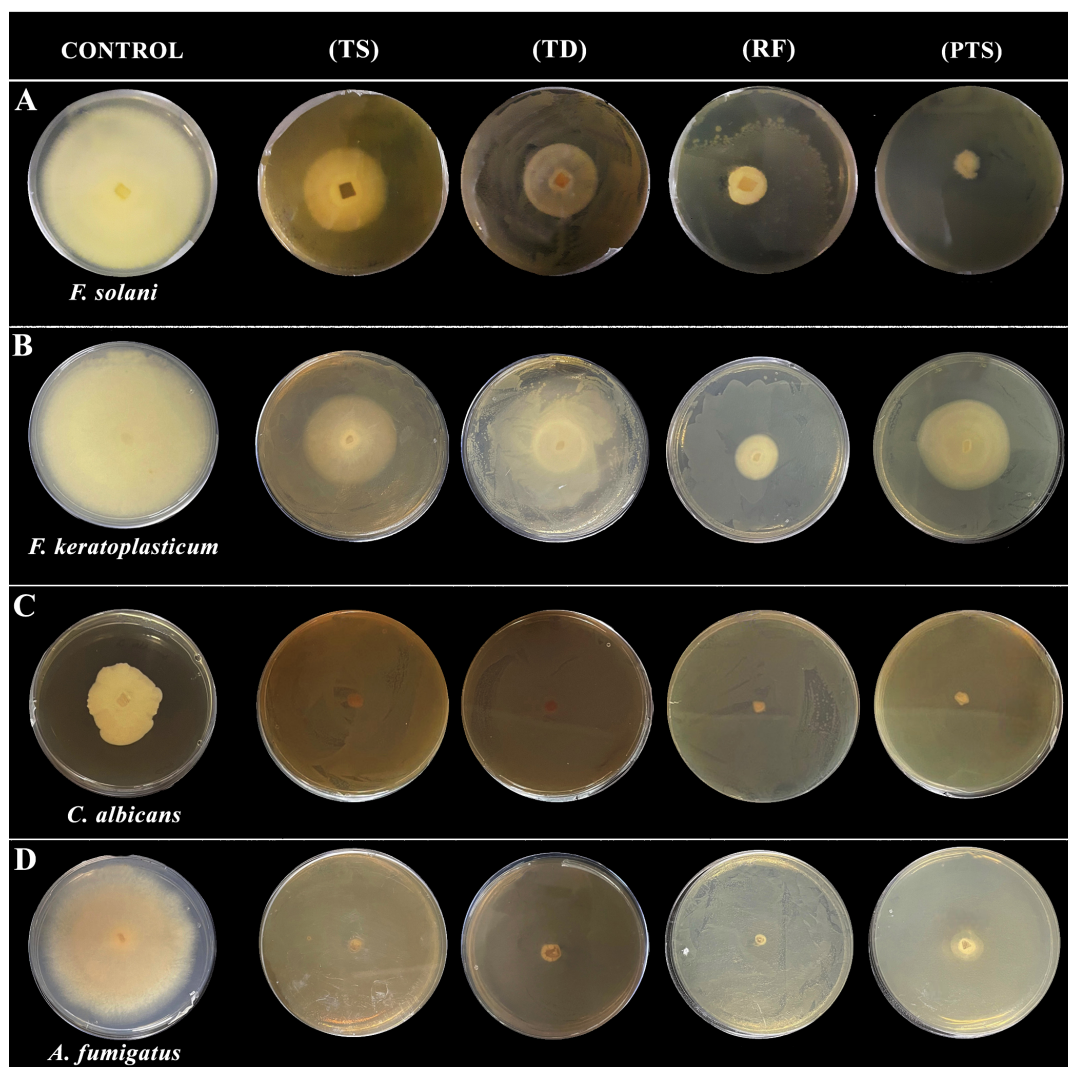


Figure 1. Growth inhibition of cell suspension of bacterial isolates against the pathogenic fungi. A (*F. solani*), B (*F. keratoplasticum*), C (*C. albicans*), and D (*A. fumigatus*).

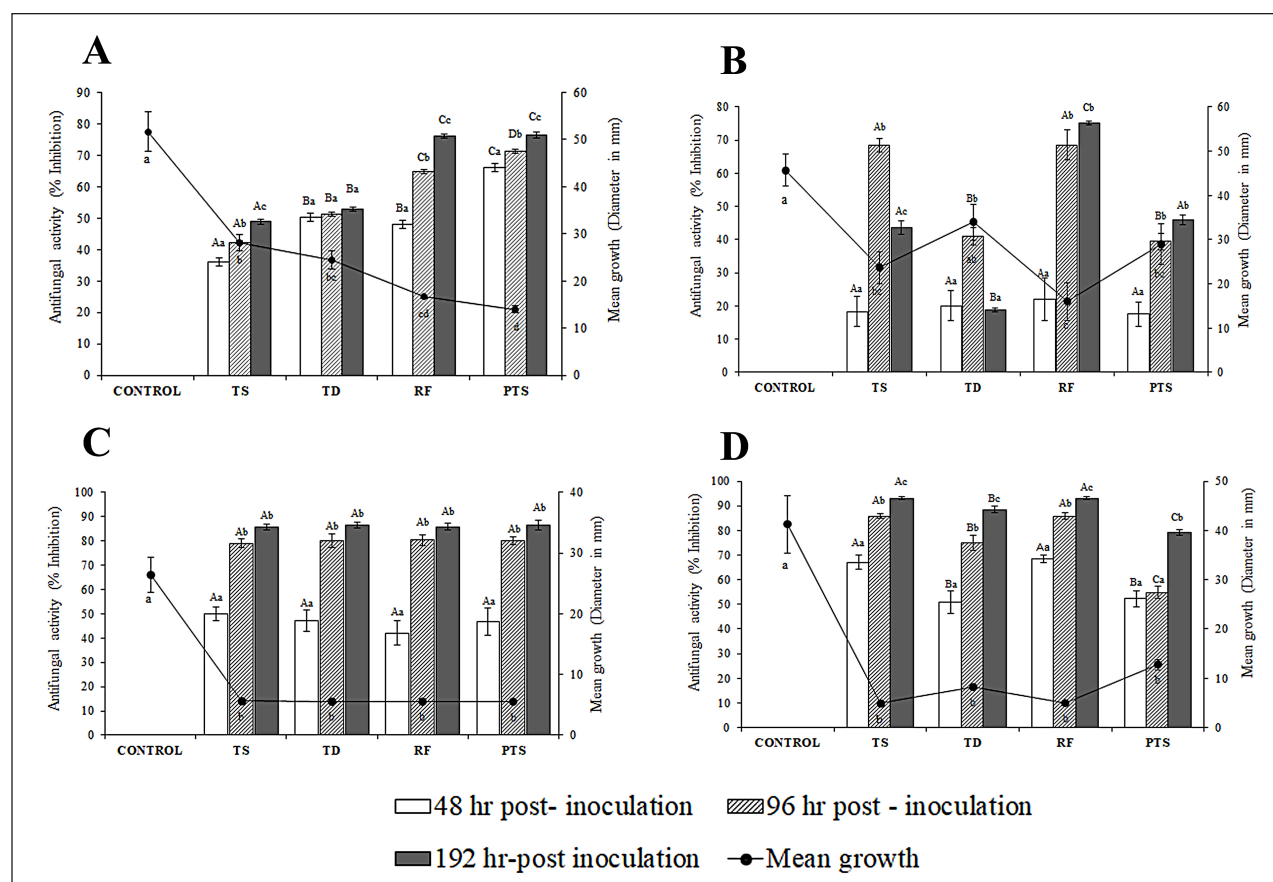


Figure 2. Graphical representation of inhibition activity of bacterial cell suspension against pathogenic fungi. The results were presented as growth inhibition (%) $\pm$ SEM and mean growth diameter (Mean $\pm$ SEM). Bars with uppercase letters indicate significant differences ($p < 0.05$) in % inhibition of different isolates at the same incubation time. Bars with different lowercase letters indicate significant differences ($p < 0.05$) in % inhibition of each isolate at different incubation times (hour) and the mean growth of fungus. A (*F. solani*), B (*F. keratoplasticum*), C (*C. albicans*), and D (*A. fumigatus*).

post-inoculation. A higher rate of percent inhibition was observed with *X. vietnamensis* (RF) and *X. stockiae* (PTS) isolates with growth inhibition of 76.04 % and 75.85%, respectively. *P. namnaonensis* (TD) isolate did not show significant inhibition activity against *F. solani* at different observation times ($p > 0.05$), while the other isolates exhibited significant differences at different observation times ($p < 0.05$).

In *F. keratoplasticum*, a significant variation of activity was observed with all the bacterial isolates ($df = 3$ and 68 ; $F = 5.54$; $p < 0.05$). *Photorhabdus hindustanensis* (TS) exhibited a significant increase in growth inhibition until 96 hours of incubation with 68.40% followed by a decline in growth inhibition at 192 hours (43.71%). Similarly, an increase in growth inhibition of 41.04% was observed at 96 hours in *P. namnaonensis* (TD) with a slight decrease of 18.81% inhibition at 192-hour incubation. A significant rise in percent inhibition was observed with *X. vietnamensis* (RF) during 192-hour incubation with 74.17%. However, a comparatively lower inhibition percentage was observed with *X. stockiae* (PTS) with a percent inhibition of 45.93% at 192-hour incubation. All the isolates showed significant growth inhibition within the first two observation periods ($p < 0.05$). However, the two *Xenorhabdus* isolates (RF and PTS) did not show significant growth inhibition from 48- to 192-hour incubation ($p > 0.05$).

In *C. albicans*, all the isolates showed a rise in percent inhibition during the observation period. However, there is no significant variation

of activity among the bacterial isolates ($df = 3$ and 68 ; $F = 0.04$; $p > 0.05$). Both *P. hindustanensis* (TS) and *X. vietnamensis* (RF) exhibited an increase in percent inhibition with 85.66% at 192 hours incubation. Also, *P. namnaonensis* (TD) and *X. stockiae* (PTS) isolates showed an insignificant percent inhibition of 86.49% and 86.48%, respectively. This study exhibited high effectiveness of bacterial isolates against *C. albicans*. Significant growth inhibition was observed in all the bacterial isolates between 48 hours and 96 hours ($p < 0.05$). However, there is no significant increase in growth inhibition at 192 hours ($p > 0.05$).

Aspergillus fumigatus growth was suppressed by all the bacterial isolates within the observation period with significant variation ($df = 3$ and 68 ; $F = 8.58$; $p < 0.05$). All the isolates exhibited an increase in percent inhibition during the different incubation times. Both *P. hindustanensis* (TS) and *X. vietnamensis* (RF) isolates showed an insignificant percent inhibition of 93.24% and 93.46%, respectively, at 192-hour post-inoculation. *P. namnaonensis* (TD) and *X. stockiae* (PTS) isolates exhibited a rise in percent inhibition of 88.50% and 79.04%, respectively, at 192 hours of incubation. *P. hindustanensis* (TS), *P. namnaonensis* (TD), and *X. vietnamensis* (RF) showed significant differences in percent inhibition of growth within the different observation times ($p < 0.05$). However, there is no significant growth inhibition in *X. stockiae* (PTS) within 48–96-hour incubation period ($p > 0.05$). Furthermore, the comparison of mean growth between the control and bacterial isolates exhibited a

Table 1. List of compounds identified in ethyl acetate extract of bacterial isolates.

Ethyl acetate extract of bacterial Isolates											
Sl. No	Compound	Molecular formula	Molecular weight (Da)	TS		TD		RF		PTS	
				Relative abundance (%)	RT	Relative abundance (%)	RT	Relative abundance (%)	RT	Relative abundance (%)	RT
1	Benzeneacetic acid (Phenylacetic acid)	C ₈ H ₈ O ₂	136	7.87	18.83	8.92	19.79	21.44	18.99	10.78	Antibacterial and antifungal [55,56]
2	2-Propenoic acid, 3-phenyl-, (E)- (trans-cinnamic acid)	C ₉ H ₁₀ O ₂	150	2.12	20.72	2.11	21.28	4.46	20.77	2.4	Antibacterial and antifungal [57]
3	(Z)-3-Phenyl-2-propenoic acid (cis-cinnamic acid)	C ₉ H ₈ O ₂	148	4.97	24.22	NA	NA	NA	NA	NA	Antibacterial, antifungal antioxidant [57,58]
4	Tetradecanoic acid (Myristic acid)	C ₁₄ H ₂₈ O ₂	228	5.9	32.17	3.96	NA	NA	32.13	1.67	Antifungal, antioxidant, nematocidal, and larvicidal [59,60]
5	Pentadecanoic acid (Pentadecylic acid)	C ₁₅ H ₃₀ O ₂	242	2.22	NA	NA	NA	NA	NA	NA	Antibacterial, antifungal, and anti-inflammatory [61,62]
6	9-Octadecenoic acid (Z)-, methyl ester (Oleic acid)	C ₁₉ H ₃₆ O ₂	296	1.68	NA	NA	37.84	1.3	37.83	5.86	Antibacterial and antifungal [63–65]
7	9-Hexadecenoic acid, (Z) (Palmitoleic acid)	C ₁₆ H ₃₀ O ₂	254	2.16	NA	NA	NA	NA	NA	NA	Antibacterial, antifungal [66,67]
8	n-Hexadecanoic acid (Palmitic acid)	C ₁₆ H ₃₂ O ₂	256	9.68	35.11	16.46	NA	NA	35.21	18.27	Antibacterial, antifungal, and anti-inflammatory [61,62]
9	Piperidine, 1-(cyanoacetyl)-	C ₈ H ₁₂ N ₂ O	152	1.36	35.48	0.72	NA	NA	NA	NA	Unknown
10	9-Octadecenoic acid, (E)- (Elaidic acid)	C ₁₈ H ₃₄ O ₂	282	3.97	36.11	1.56	NA	NA	36.15	1.56	Antibacterial, antifungal, and antioxidant [68]
11	Pyrrolo [1, 2-a] pyrazine-1, 4-dione, hexahydro-3-(2-methylpropyl)	C ₁₁ H ₁₈ N ₂ O ₂	210	NA	34.82	13.53	35.21	5.74	34.86	11.6	Antimicrobial, antifungal, and antioxidant [69,70]
12	2,5-Piperazinedione, 3, 6-bis(2-methylpropyl)-	C ₁₂ H ₂₂ N ₂ O ₂	226	1.29	39.23	2.24	40.21	4.94	39.44	0.82	Antifungal [71,72]
13	Pyrrolo [1, 2-a] pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	C ₁₄ H ₁₆ N ₂ O ₂	244	5.85	41.46	10.57	41.98	4.62	41.49	11.82	Antimicrobial, antifungal, and antioxidant [69,70]
14	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester	C ₁₉ H ₃₈ O ₄	330	1.21	42.91	1.43	42.98	1.2	42.91	0.93	Antioxidant, pesticidal, and nematocidal [73]
15	Bis (2-ethylhexyl) phthalate (Phthalic acid)	C ₂₄ H ₃₈ O ₄	390	1.3	43.05	0.92	43.09	1.5	NA	NA	Antibacterial, antifungal, and insecticidal [74,75]

(Continued)

Ethyl acetate extract of bacterial Isolates												
Sl. No	Compound	Molecular formula	Molecular weight (Da)	TS		TD		RF		PTS		
				Relative abundance (%)	RT	Relative abundance (%)	RT	Relative abundance (%)	RT	Relative abundance (%)	RT	
16	L-Phenylalanyl-L-leucine	C ₁₅ H ₂₂ N ₂ O ₃	278	2.96	44.29	2.61	44.24	44.45	1.98	44.22	2.17	Unknown
17	13-Docosenamide, (Z) (Erucamide)	C ₂₂ H ₄₃ NO	337	2.79	45.53	5.91	45.53	45.56	2.15	45.52	3.71	Antibacterial and antifungal [76,77]
18	2,5-Piperazinedione, 3-(phenylmethyl)-	C ₁₁ H ₁₂ N ₂ O ₂	204	16.9	45.96			45.68	1.45			Unknown
19	Cyclo (L-prolyl-L-valine)	C ₁₀ H ₁₆ N ₂ O ₂	196	NA	NA	9.49	33.09	33.35	16.23	33.1	8.48	Antifungal [78]
20	3,6-Diisopropylpiperazin-2,5-dione	C ₁₀ H ₁₈ N ₂ O ₂	198	NA	NA	NA	NA	34.67	5.77	34.26	2.31	Antimalarial [79]
21	Benzeneethanol, 4-hydroxy	C ₈ H ₁₀ O ₂	138	NA	NA	NA	NA	23.23	2.91	NA	NA	Antibacterial and antioxidant [80]
22	2-Propenoic acid, hexadecyl ester	C ₁₉ H ₃₆ O ₂	296	NA	NA	NA	NA	30.77	0.42	NA	NA	Unknown
23	Benzo[h]quinoline, 2,4-dimethyl	C ₁₅ H ₁₃ N	207	NA	NA	NA	NA	32.2	0.43	NA	NA	Antibacterial and antifungal [81]
24	N, N, N', N'-Tetraethyl-2-di-furan-2-yl-ethane-1, 2-diamine	C ₁₈ H ₂₈ N ₂ O ₂	304	NA	NA	NA	NA	35.77	1.39	NA	NA	Unknown
25	Benzoic acid, 4-butoxy-3-methoxy-, perhydro-1-quinolizinylmethyl ester	C ₂₂ H ₃₃ NO ₄	375	NA	NA	NA	NA	NA	NA	35.54	0.79	Unknown
26	4,8,12,16,20,24,28-Heptamethyl-1,5,9,13,17,21,25-hepta-oxa-cyclooctacosane-2,6,10,14,18,22,26-heptaone	C ₂₈ H ₄₂ O ₁₄	602	NA	NA	NA	NA	NA	NA	37.46	1.97	Unknown

* TS (*P. hindustanensis*), TD (*P. namnaonensis*), RF (*X. vietnamensis*), PTS (*X. stockiae*) RT (Retention time), NA (Not-applicable).

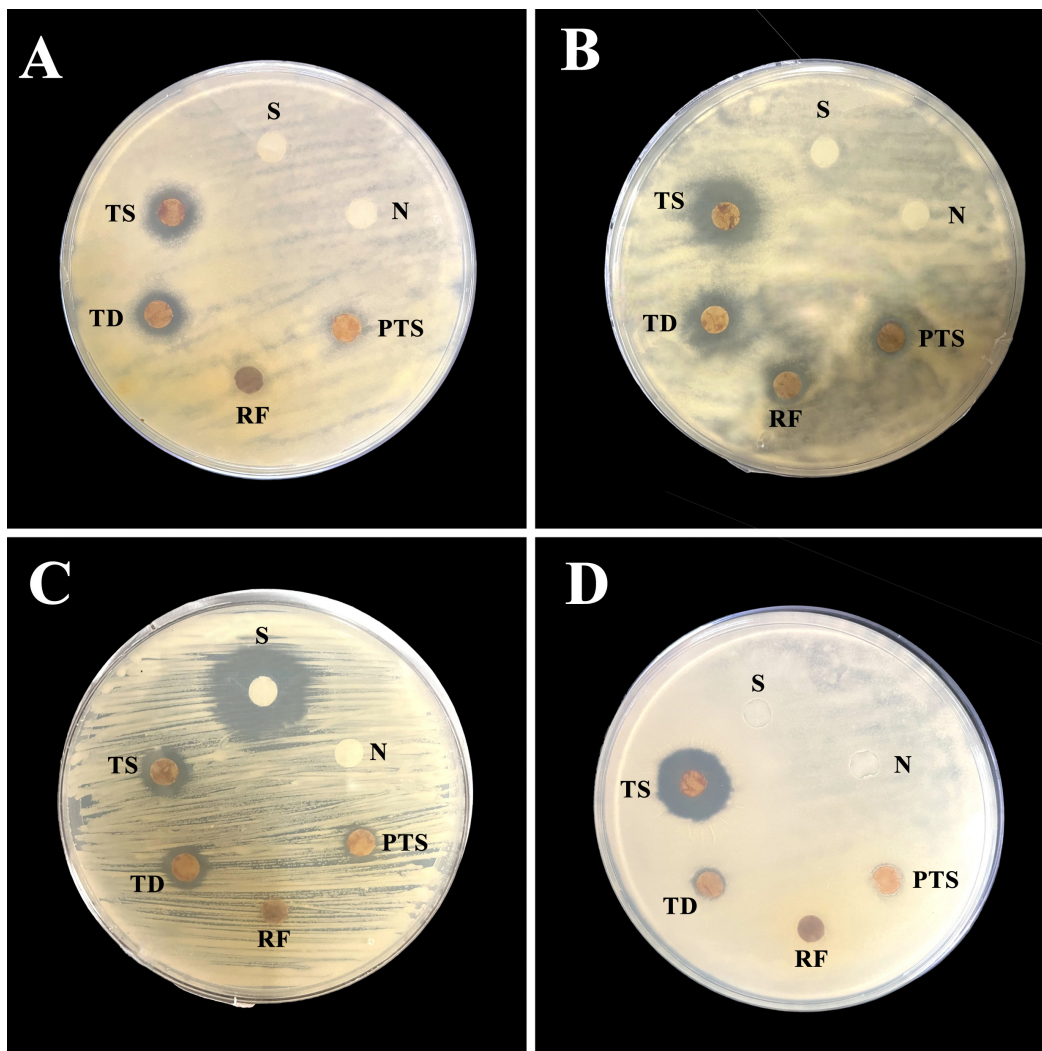


Figure 3. Antifungal activity of ethyl acetate extract of bacterial isolates against pathogenic fungi. (A) *F. solani*, (B) *F. keratoplasticum*, (C) *C. albicans*, and (D) *A. fumigatus*.

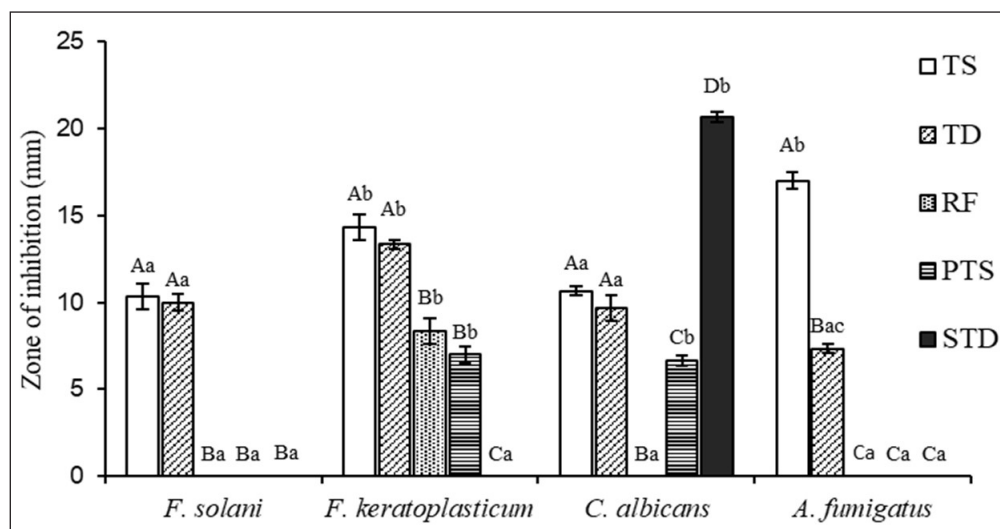


Figure 4. Graphical representation of inhibition activity of ethyl acetate extract of bacterial isolates against pathogenic fungi. The results were presented as mean growth diameter (Mean $\pm$ SEM). Bars with different uppercase indicate significant differences ($p < 0.05$) of different isolates against each pathogenic fungus. Lowercase letters indicate significant differences ($p < 0.05$) of each bacterial isolate against different pathogenic fungi.

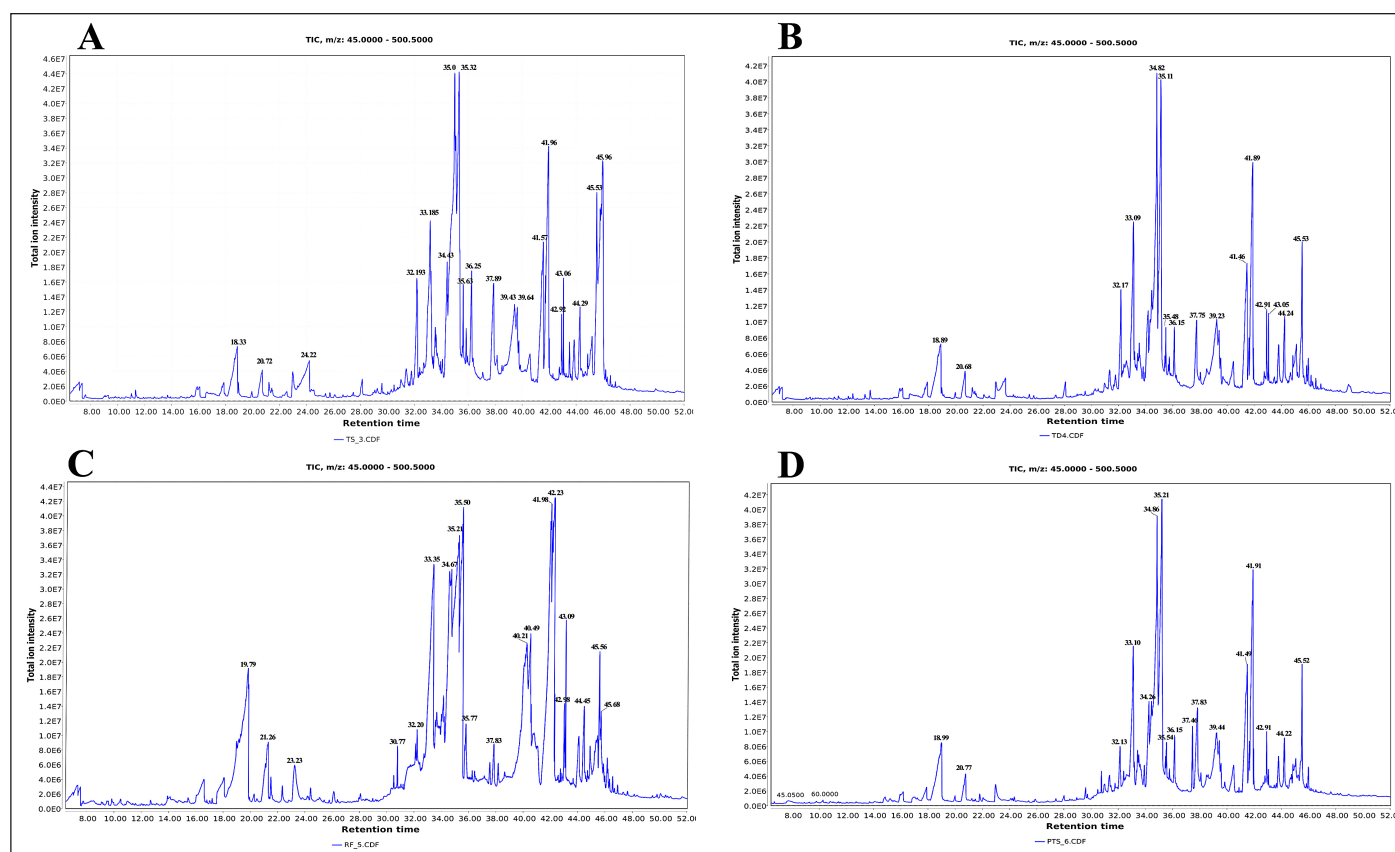


Figure 5. GC-MS chromatogram of the components in the ethyl acetate extract of bacterial isolates. A (*P. hindustanensis* TS), B (*P. namnaonensis*TD), C (*X. vietnamensis*, RF), and D (*X. stockiae*, PTS).

significant variation during the observation period ($p < 0.05$) in all the pathogenic fungi.

3.2. Antifungal Activity of Ethyl Acetate Extract

The ethyl acetate extracts of bacterial isolates, including the standard (fluconazole), exhibited significant variations of activity against each pathogenic fungus, i.e., *F. solani* ($df = 4$ and 10 ; $F = 139.6$; $p < 0.05$), *F. keratoplaticum* ($df = 4$ and 10 ; $F = 82.41$; $p < 0.05$), *C. albicans* ($df = 4$ and 10 ; $F = 252.40$; $p < 0.05$), and *A. fumigatus* ($df = 4$ and 10 ; $F = 631$; $p < 0.05$). Among the bacterial isolates, the extract of TS isolate was found to be most potent where it inhibited the growth of the tested pathogenic fungi with an inhibition zone of 10.33–17 mm diameter. The extract of *P. namnaonensis* (TD) showed a clear zone of inhibition against *F. solani*, *F. keratoplaticum*, and *C. albicans* with the highest being recorded with *F. keratoplaticum* (13.33 mm diameter). However, the extract of *Xenorhabdus* (RF and PTS) isolates showed significantly lower activity than the *Photorhabdus* (TS and TD). The extract of *X. vietnamensis* (RF) isolate only inhibited the growth of *F. keratoplaticum*, with 8.33 mm diameter of inhibition zone. The extract of *X. stockiae* (PTS) exhibited comparatively lower activity compared to the other isolates against the pathogenic fungi, exhibiting negligible inhibitory activity against *F. keratoplaticum* and *C. albicans* with 7 mm and 6.67 mm diameter, respectively. The standard disc of fluconazole (10 μ g) showed inhibition activity against *C. albicans* alone, with 20.67 mm in diameter. The highest inhibition of bacterial extract against the pathogenic fungi was observed with *A. fumigatus* (ATCC) where *P. hindustanensis* (TS) isolate showed a clear inhibition zone of 17 mm in diameter. The growth inhibition of bacterial extracts

against fungal growth and the graphical representation of inhibitory activity are shown in Figures 3 and 4.

3.3. GCMS Analysis of Ethyl Acetate Extract of Bacterial Isolates

A total of 26 different compounds were characterized, in which 20, 16, 20, and 17 peaks were exclusively analyzed from *P. hindustanensis* (TS), *P. namnaonensis* (TD), *X. vietnamensis* (RF), and *X. stockiae* (PTS), respectively (Fig. 5). The lists of the corresponding compounds with their retention time, relative abundance (%), molecular formula, molecular weight (Da), and biological activity were given in Table 1.

The most prevalent compound in the *P. hindustanensis* (TS) isolate was found to be [Pyrrolo(1,2-a) pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)], which was observed at two peaks with a relative abundance of 17.64% and 5.85%. A considerable amount of 2,5-Piperazinedione, 3-(phenylmethyl), a saturated long chain fatty acid (n-Hexadecenoic acid), and benzenecetic acid were present with a relative abundance of 16.9%, 9.68%, and 7.87%, respectively.

The most abundant compound in *P. namnaonensis* (TD) isolate was found to be n-Hexadecanoic acid, which had a relative abundance of 16.46%, followed by [Pyrrolo (1,2-a) pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)], which had two peaks with 15.64% and 10.57% relative abundances. Cyclo (L-prolyl-L-valine) and benzenecetic acid were also detected in moderate proportion, with relative abundances of 9.49% and 8.92%, respectively.

Benzenecetic acid was discovered to be the most abundant compound in the *Xenorhabdus vietnamensis* (RF) isolate, accounting for 21.44%

of the total relative abundance. In addition, a notable abundance of diketopiperazine compounds, such as Cyclo (L-prolyl-L-valine), Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(phenylmethyl), and 3,6-Diisopropylpiperazin-2,5-dione, were present, which exhibited 16.23%, 10.95%, 8.87%, and 5.77% relative abundance, respectively.

In *X. stockiae* (PTS) isolate, a saturated long-chain fatty acid, n-Hexadecanoic acid was the most abundant compound with 18.27% relative abundance. Other compounds that were reasonably abundant were Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(phenylmethyl), which showed two peaks with relative abundance of 14.86% and 11.82%. A modest amount of Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), benzeneacetic acid, and Cyclo(L-prolyl-L-valine) were also detected with relative abundance of 11.60%, 10.78%, and 8.48%, respectively.

Among the available compounds detected by GCMS, the common chemical compounds found in all the isolates were benzeneacetic acid, 2-Propenoic acid, 3-phenyl-, (E)- (trans-cinnamic acid), 2,5-Piperazinedione, 3,6-bis(2-methylpropyl), Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(phenylmethyl), Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester, L-Phenylalanyl-L-leucine, and 13-Docosenamide, (Z) with a compound belonging to the class of diketopiperazine being the most abundant.

4. DISCUSSION

Natural products (NPs) have sufficient structural intricacy and scaffold variety. In the recent decade, NPs and their analogs have made significant contributions to various pharmaceutical industries. Besides this, NPs face various challenges such as insufficient methods for isolation, characterization, screening, and optimization, which decline their pursuit of further drug discovery [38].

A wide range of small compounds produced by *Photobacterium* and *Xenorhabdus* spp. are known to possess several biological functions in both the mutualistic and pathogenic phases [39,40]. It was revealed that the bioactive compounds extracted from the fermented culture media of *Photobacterium* spp. and *Xenorhabdus* spp., such as methanol and ethyl acetate, were highly effective in controlling a variety of pathogenic bacteria [27–29,33]. Furthermore, these bioactive chemicals have been shown to exhibit a potential biological activity against insect pests, parasites, and cancer cells [25,41,42]. The pathogenicity of *Photobacterium* and *Xenorhabdus* against several fungal pathogens was also investigated using a variety of techniques that involved contact with cell suspension, cell-free supernatant, and solvent extract as well as VOCs with a high success rate. [30–32,43–50].

The cell-free filtrate and methanol extract of *X. bovienii* successfully inhibited the growth of *B. cinerea* and *P. capsici* [46]. Similarly, effective natural products from *X. budapestensis* and *X. szentirmaii* reduced *P. nicotianae* colony formation and mycelial development [47]. Furthermore, Cimen et al. [45] evaluated the activity of *Xenorhabdus* spp. and *Photobacterium* spp. against *C. parasitica*, *F. oxysporum*, *R. solani*, and *S. sclerotiorum* and concluded that the *Xenorhabdus* spp. were considerably more active than the *Photobacterium* in inhibiting the fungal pathogens. However, in this study, variations of antagonistic activity of the bacterial isolates were observed against different fungal pathogens, which is consistent with the observation of Ulug [48] where *X. cabanillasii* and *X. szentirmaii* exhibited significant suppression of fungal growth. The variations among different studies may be attributed to the degree of interaction of each bacterial isolate against different fungus and culture media used for the study [39,45].

Based on the co-culturing approach analyzed in this study, a highly significant inhibitory activity of all the bacterial isolates against *A. fumigatus* and *C. albicans* was observed. *P. hindustanensis* (TS) isolates showed moderate inhibition against *F. solani* and *F. keratoplasticum*. Similarly, *P. namnaonensis* (TD) isolate caused a fair inhibition of *F. solani* but did not inhibit the mycelial growth of *F. keratoplasticum*. Chen et al. [30] observed that the antifungal activity of phase two *Photobacterium* spp. and *Xenorhabdus* spp. were significantly weak. It is worth mentioning that we observed the same trend where the inhibition rate of *Photobacterium* isolates (TS and TD) diminished over time against *F. keratoplasticum*. However, *Xenorhabdus* isolates (RF and PTS) showed an increase in the percent inhibition rate against *F. solani* and *F. keratoplasticum* during the different observation periods. Also, Lalramchuan et al. [49] observed a significant inhibition of *X. vietnamensis* against *F. oxysporum* by a co-culturing approach.

Antifungal assay, ethyl acetate extract, of *P. hindustanensis* (TS) showed a high inhibition activity against *A. fumigatus* and a moderate inhibition activity against *F. solani*, *F. keratoplasticum*, and *C. albicans*. However, *P. namnaonensis* (TD) showed moderate inhibition activity against *F. solani*, *F. keratoplasticum*, and *C. albicans*, but a very low activity was observed against *A. fumigatus*. The ethyl acetate extract of *X. vietnamensis* (RF) and *X. stockiae* (PTS) exhibited minimal or no inhibition activity against all pathogenic fungi. In contrast, Xenocoumacin 1, derived from *Xenorhabdus nematophila*, exhibits a broad antifungal spectrum against *S. sclerotiorum*, affecting fungal morphology as well as enzymatic activity [50]. The occurrence of these variations in the inhibition activity between the two genera might be attributed to the difference in the active chemical compounds present in the extract of each bacterial isolate [32,45,46].

A variety of compounds with known antifungal properties were characterized using the GCMS analysis from each bacterial extract. Most of the compounds present in the ethyl acetate extract of the bacterial isolates were found to be fatty acid derivatives and peptide compounds. It is noteworthy that the presence of a well-known antifungal compound transcinammic acid (TCA) was observed in the ethyl acetate extract of *P. namnaonensis* (TD), *X. vietnamensis* (RF), and *X. stockiae* (PTS) isolates, while the cinnamic acid in a cis-form was also observed in *P. hindustanensis* (TS) isolate. Hazir et al. [51] evaluated the potency of four *Xenorhabdus* spp. and three *Photobacterium* spp. against *Fusarium carpophilum*, *F. effusum*, *Monilinia fructicola*, *Glomerella cingulata*, and *Armillaria tabescens*. They compare the efficacy of the bacterial metabolites that were previously reported as bioactive compounds of *Photobacterium luminescens* [52]. They observed that TCA was the most effective treatment with significant variation. In addition, cinnamic acids offer a novel method of action since they target enzymes specific to fungus and can be used as lead compounds in the design and production of new medications with less harmful effects on higher eukaryotes. Korosec et al. [53] and Sa-Uth et al. [54] revealed a high efficacy (98.62%) of *X. stockiae* against several plant pathogenic fungi, including *Fusarium* sp. by enhancing the composition of medium supplemented with sucrose, yeast extract, NaCl, and K₂HPO₄.

5. CONCLUSION

This study highlights the potential of the antifungal activity of *Photobacterium* and *Xenorhabdus* against several pathogenic fungi. While the antifungal properties of these bacteria are promising, further research is necessary to fully understand their mechanisms and optimize their application in agricultural settings. The potential for resistance development in pathogens remains a concern, necessitating ongoing evaluation of these biocontrol agents. These extracted metabolites could be potent antifungal agents to combat

certain pathogenic fungi, which become resistant to the currently available antifungal drugs. Therefore, the information provided in this study will pave the way for further examination of certain beneficial microorganisms including their metabolites for the treatment of different diseases worldwide. Since entomopathogenic bacteria could be a promising alternative agent to combat the current global crisis of antifungal resistance, more efforts and detailed analysis, including appropriate formulations, must be investigated for effective use to control several fungal pathogens.

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7. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. DATA AVAILABILITY

All the data are available with the authors and will be provided upon request.

10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

11. PUBLISHER'S NOTE

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12. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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